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GLUTAMATE RECEPTORS OF GANGLION CELLS IN THE RABBIT RETINA: EVIDENCE FOR GLUTAMATE AS A BIPOLAR CELL TRANSMITTER

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SUMMARY

1. Intracellular and extracellular recordings were obtained from ganglion cells in the rabbit retina. The effects of glutamate analogues and antagonists were studied using a perfusion method for drug application.

2. Kainate (KA) excited all ganglion cells directly and caused a large increase in firing rate. *N*-Methyl-DL-aspartate (NMDLA) also excited ganglion cells but it was less potent and caused burst firing.

3. Quisqualate (QQ) and (*RS*)-2-amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid (AMPA) excited many ganglion cells and were approximately as potent as KA. Less frequently, QQ and AMPA had inhibitory effects possibly due to polysynaptic action.

4. General glutamate antagonists such as cis-2,3-piperidine dicarboxylic acid (PDA) and kynurenic acid blocked the light input to all ganglion cells. PDA and kynurenic acid blocked the effects of KA and NMDLA, but not carbachol, indicating that they act as glutamate antagonists in the rabbit retina. Kynurenic acid did not block the excitatory action of QQ, even though light responses were abolished.

5. Amacrine cells were depolarized by KA or QQ and less potently by NMDLA. Their light-evoked responses were blocked by PDA.

6. We conclude that the light input to ganglion cells in the rabbit retina is predominantly mediated by KA receptors. This is consistent with the idea that 'on' and 'off' bipolar cells are excitatory and release glutamate.

INTRODUCTION

Recent evidence indicates that glutamate is a major excitatory neurotransmitter in the vertebrate central nervous system (Mayer & Westbrook, 1987). Like other neurotransmitters, glutamate interacts with a variety of postsynaptic receptors which have been classified by their differential affinities for specific glutamate analogues. Thus they are commonly referred to as (i) *N*-methyl-D-aspartate (NMDA)

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receptors, (ii) kainate (KA) receptors, (iii) quisqualate (QQ) receptors and (iv) 2-amino-4-phosphonobutyrate (APB) receptors after the specific analogues which activate them (Foster & Fagg, 1984). All these glutamate receptor subtypes have been reported in the retina (Miller & Slaughter, 1986; Coleman, Massey & Miller, 1986).

The vertebrate retina is a layered structure containing six major neuronal classes. In the simplest terms, visual information passes from photoreceptors to horizontal and bipolar cells and then, via bipolar cells, to amacrine and ganglion cells. Thus, bipolar cells are the principal elements which transmit light-evoked activity from the outer retina to the inner retina. Physiological evidence obtained from current injection into bipolar cells (Naka, 1977), transretinal stimulation (Toyoda & Fujimoto, 1984) or selectively blocking 'on' bipolar cells (Miller, 1979; Slaughter & Miller, 1981) indicates that bipolar cells are excitatory. In addition, the postsynaptic structures at mammalian bipolar cell ribbon synapses are typical of other excitatory synapses (Raviola & Raviola, 1982). However, the identity of a neurotransmitter for bipolar cells has not yet been established.

In the rabbit retina, rod and cone pathways are segregated via separate rod and cone bipolar cells. Cone signals are carried by 'on' and 'off' bipolar cells which synapse directly with 'on' and 'off' ganglion cells respectively. In contrast, rod bipolar cells rarely make direct contact with ganglion cells. Instead, rod signals pass via intermediary amacrine cells (Kolb & Nelson, 1984). Rod bipolar cells and their principal postsynaptic targets, AII and A17 amacrine cells, all depolarize to light (Dacheux & Raviola, 1986, 1987). Therefore, rod bipolar cells are also excitatory, but their connections are not specifically considered in this paper.

Glutamate receptors are abundant in both the outer and inner retina. There is strong evidence, derived from intracellular recording of second-order neurones, that photoreceptors release glutamate (Lasater & Dowling, 1982; Ishida, Kaneko & Tachibana, 1984; Miller & Slaughter, 1986). Convincingly, glutamate produces different postsynaptic responses by interacting with a variety of postsynaptic receptors. Thus, horizontal and 'off' bipolar cells are depolarized via sign-conserving KA (or QQ) receptors, but 'on' bipolar cells are hyperpolarized by the action of glutamate at a distinct sign-inverting receptor which is selectively activated by APB (Slaughter & Miller, 1981; Miller & Slaughter, 1986). This sign inversion between photoreceptors and 'on' bipolar cells underlies the generation of 'on' responses throughout the retina and the rest of the visual system. In contrast, NMDA receptors appear to be rare or absent in the outer retina (Slaughter & Miller, 1983*a*, 1985; Massey & Miller, 1987).

In the inner retina, neuronal interactions are more complex, but preliminary results indicate that, like photoreceptors, bipolar cells may release glutamate (Slaughter & Miller, 1983*c*). Third-order neurones in the mudpuppy retina are directly excited by KA, QQ and NMDLA, and their light responses are blocked by glutamate antagonists (Slaughter & Miller, 1983*b*, *c*; Lukasiewicz & McReynolds, 1985; Coleman *et al.* 1986). APB has no direct action in the inner retina and it appears that APB receptors are mostly restricted to the photoreceptor–'on' bipolar cell synapse (Slaughter & Miller, 1981, 1985). In the rabbit retina, KA, NMDLA and QQ depolarize amacrine and ganglion cells but a direct effect has not been

demonstrated (Bloomfield & Dowling, 1985). Early glutamate antagonists, such as α-methyl glutamate and α-aminoadipate, are not as effective as current antagonists but the light responses of third-order neurones are reduced or blocked by their application (Bloomfield & Dowling, 1985).

The goal of the present experiments was to examine the pharmacology of ganglion cell responses in the rabbit retina and thus, by inference, assess the characteristics of the cone bipolar cell neurotransmitter in the mammalian retina. This is directly analogous to the analysis of horizontal cell responses to identify the photoreceptor transmitter. Our results support the idea that 'on' and 'off' bipolar cells release glutamate and suggest that the light-driven input to ganglion cells is predominantly via KA receptors. Preliminary accounts of this work have been published (Massey & Miller, 1984, 1985*a*).

METHODS

Intracellular and extracellular recordings were made from the everted and perfused rabbit eye-cup which has been described in detail elsewhere (Miller, Zalutsky & Massey, 1986). Briefly, a pigmented rabbit was deeply anaesthetized with urethane (1·5 g/kg, I.P.) and the orbit was infused with xylocaine. One eye was removed, hemisected and everted over a Teflon plug threaded to accept a retaining ring which gently clamped the tissue at the periphery for stability. This apparatus was placed in an oxygenation chamber on a vibration isolation table inside a Faraday cage. Perfusion fluid flowed by gravity through an in-line heater where it was heated to 37 °C, then over the surface of the retina in a thin film. Up to twelve drug solutions could be applied to the retina with a lag time of 20 s by switching valves in a manifold above the cage. This perfusion method permits prolonged recording from single units through multiple solution changes.

Electrophysiological recording

Extracellular recordings (bandwidth 1–3 kHz) were made using tungsten electrodes (Levick, 1972). Action potentials were passed through a window discriminator and then to an analog ratemeter which proved convenient to compress large amounts of data. Occasionally, peristimulus time histograms were constructed by computer, either on-line or after play-back. Intracellular recordings were obtained using electrodes pulled from 1·2 mm Omega Dot glass filled with 3 M-potassium acetate (resistance 100–400 MΩ). The electroretinogram (ERG) was recorded between one silver–silver chloride electrode in the Teflon plug and another which served to ground the perfusate. The ERG was monitored continuously to assess the condition of the retina. Data were recorded on an FM tape deck (Vetter, Model D or 420) and a pen recorder from which the ratemeter records could be photographed directly. Records containing action potentials were photographed from a storage oscilloscope, played back at slow speed to the pen recorder or digitized by computer and plotted.

Light stimuli, of intensity 11–12 log quanta cm^{-2} s^{-1}, were provided by a triple beam light bench or, later in this series of experiments, by computer control of a video monitor focused onto the retinal surface. Ganglion cell types recorded extracellularly were identified by standard tests (Oyster, 1968; Caldwell & Daw, 1978). Brisk cells responded to fast stimuli (> 5 deg/s) and had centre–surround responses. Large field units gave centre and surround responses to diffuse light and responded to slight (0·2 log units) dimming of a diffuse stimulus. 'On–off' directionally selective ganglion cells gave 'on–off' responses to a small spot, no response to a diffuse stimulus and responded to fast stimuli. 'On' directionally selective ganglion cells gave only 'on' responses to a small spot and responded only to slowly moving stimuli (< 5 deg/s).

Solutions

The perfusion fluid was a bicarbonate-based buffer made according to Ames & Nesbett (1981), including all additives except serum. This solution was freshly prepared and saturated with 95% oxygen–5% carbon dioxide. Cobalt solutions, which are often thought to be incompatible with bicarbonate buffers, were made by first dissolving cobalt chloride (200 mM) in distilled water and then adding aliquots to the standard perfusion fluid less phosphate.

Kynurenic acid was obtained from Aldrich; AMPA was obtained from Research Biochemicals Incorporated; and PDA, QQ and 2-amino-7-phosphonoheptanoate (AP-7) were provided by Dr J. C. Watkins whom we thank for collaboration. Other drugs and reagents were obtained from Sigma. Drug solutions were made in the standard perfusion fluid adjusted to pH 7·4 with sodium hydroxide if required. All solutions were continuously bubbled with 95% oxygen–5% carbon dioxide during an experiment since we found that minor pH changes caused artifacts.

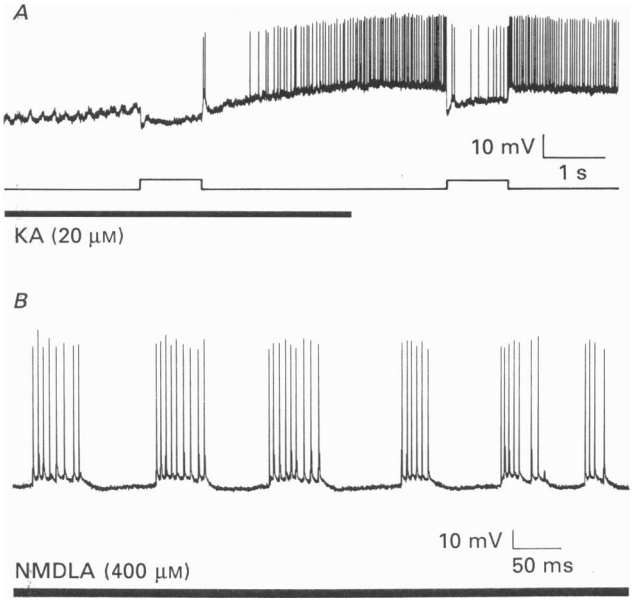

Fig. 1. *A*, an intracellular recording from an 'off' ganglion cell in the rabbit retina. The stimulus trace indicates diffuse light. Perfusion with KA for 30 s, started before this frame, caused a 10 mV depolarization and elicited a rapid burst of action potentials. Note less firing during light and a burst of action potentials at light 'off'. The resting membrane potential was −70 mV. *B*, an intracellular recording from an 'on' ganglion cell in the rabbit retina during a 30 s perfusion with NMDLA. NMDLA caused a 5 mV depolarization and distinctive burst firing. The resting membrane potential of this cell was −72 mV.

RESULTS

Glutamate is a strong candidate as an excitatory neurotransmitter in the nervous system and it has previously been shown to depolarize many neurones of the inner retina (Slaughter & Miller, 1983*b*; Bloomfield & Dowling, 1985). However, glutamate interacts with several different postsynaptic receptors and the presence of an avid uptake system requires the use of very high glutamate concentrations. In this study, we chose to use glutamate analogues which are specific for certain glutamate receptors and whose potency may be partly explained by a low affinity for the glutamate uptake carrier (Ariel, Lasater, Mangel & Dowling, 1984).

Agonists

Without exception, KA and NMDLA excited all ganglion cells in the rabbit retina including brisk, sluggish and directionally selective types. Intracellular recording (Fig. 1*A*) revealed that 20 μM-KA caused a depolarization of approximately 10 mV

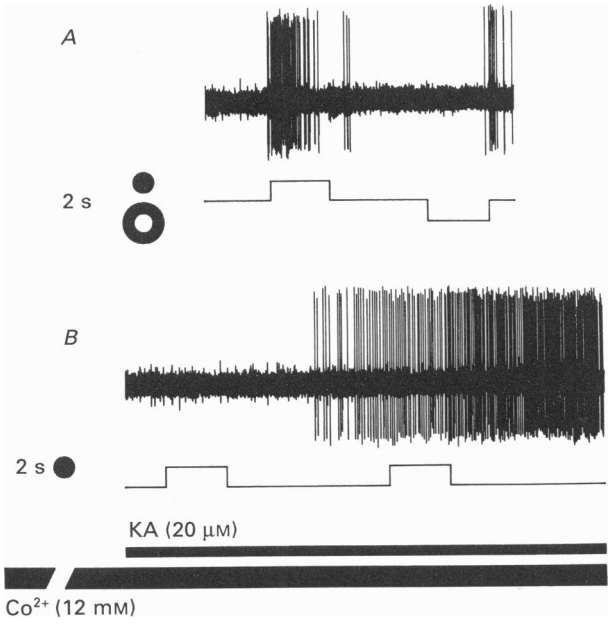

Fig. 2. *A*, an extracellular recording showing control light responses from a brisk 'on' cell. A small spot (stimulus trace up) elicited a strong 'on' response and an annulus (stimulus trace down) elicited a slower and weaker surround response at the offset. *B*, after cobalt was added to the perfusate, there was no response to stimulation with a small spot indicating the block of synaptic transmission across the retina. Under these conditions, perfusion with KA elicited a continuous chain of action potentials indicating that KA has a direct excitatory effect on ganglion cells. The time scale is indicated by the duration of the light stimulus, 2 s.

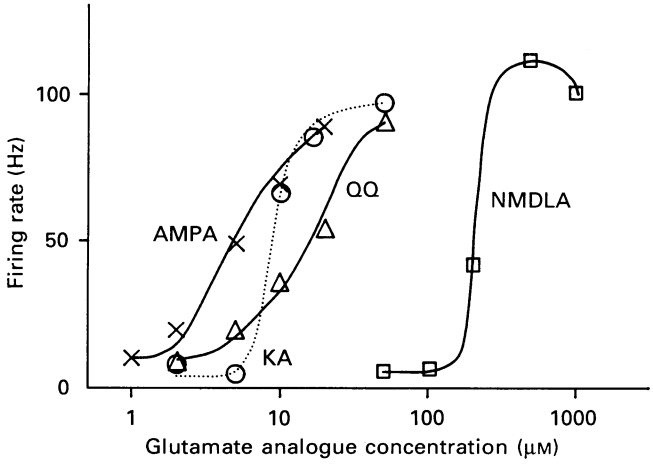

Fig. 3. Dose–response curves for the excitatory action of some glutamate analogues on rabbit ganglion cells. The curve for KA is shown as a dotted line for clarity. Peak firing rates were measured from a ratemeter trace which was calibrated against a square wave stimulator. The data for AMPA (×), QQ (△) and KA (○) were all derived from the same large field unit. The data for the NMDLA (□) curve were derived from a separate large field unit. These curves are representative of results from many different ganglion cells including 'on', 'off', sluggish, and directionally selective cells. All curves were fitted by eye.

associated with a large increase in firing rate. In particular, it may be noted that KA elicited a regular train of action potentials whose frequency increased with depolarization. This stands in direct contrast to the effect of NMDLA which caused rabbit ganglion cells to fire in a distinctive bursting pattern (Fig. 1*B*). Intracellular analysis of NMDLA-induced excitation revealed that each burst of five to ten action

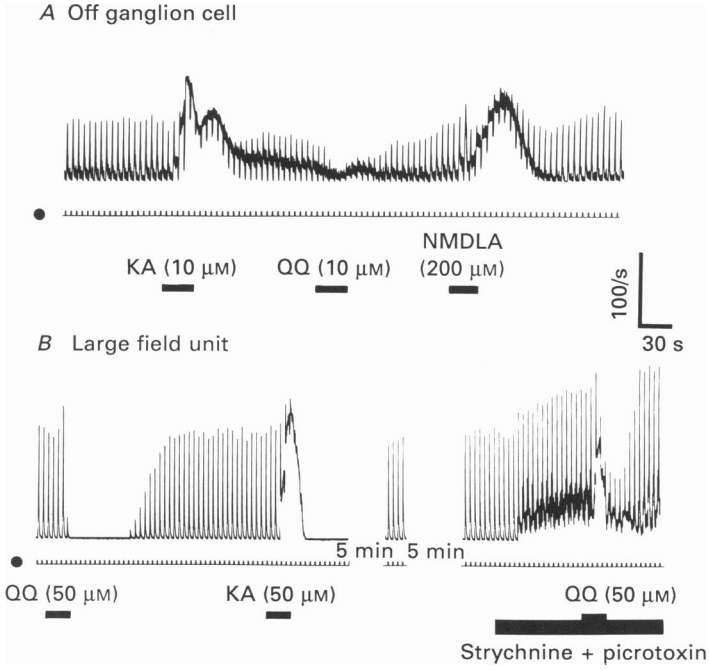

Fig. 4. *A*, a ratemeter record from a brisk transient 'off' cell. The stimulus was a small spot. KA and NMDLA both excited this cell causing a large increase in firing rate. In contrast, QQ reduced the spontaneous activity and inhibited the light-evoked responses. The calibration bar applies to both traces and indicates the cell's firing rate in action potentials per second and the time scale. *B*, a ratemeter record from a large field unit. The stimulus was a small spot. QQ inhibited the light-evoked responses of this cell. In contrast, KA caused a large increase in firing rate with a subsequent decrease in spike amplitude as the cell entered a depolarizing block. Due to the high dose of KA used in this experiment recovery was prolonged, indicated by a 5 min break in the trace. Perfusion with 20 μM-picrotoxin and 5 μM-strychnine enhanced both the spontaneous and light-evoked activity of this cell. Under these conditions, QQ excited the cell and caused a large increase in firing rate. Recovery was not obtained due to the prolonged effect of strychnine. Calibration as in *A*.

potentials rides on a small oscillation in the membrane potential. Thus, the action of NMDLA is distinct from KA and not typical of light-driven activity.

The action of KA is widespread in the retina so it was important to demonstrate a direct action on ganglion cells. Perfusion with 2 mM-cobalt eliminated the light-evoked response of the 'on' ganglion cell illustrated in Fig. 2, indicating the blockade of synaptic transmission across the retina. This is supported by previous work in the rabbit showing that 2 mM-cobalt eliminates the light responses of horizontal cells in the distal retina (Massey & Miller, 1987). The addition of 20 μM-KA during perfusion

with cobalt caused a continuous chain of action potentials independent of the light stimulus. This indicates that KA has a direct effect on ganglion cells and therefore that KA receptors are present on ganglion cell membranes.

KA is an extremely potent glutamate analogue with a threshold dose of 1–2 μM reaching saturation by 20–50 μM. Dose–response curves showed that KA is approximately 20 times more potent than NMDLA on rabbit ganglion cells (Fig. 3). At a concentration of 50 μM (and sometimes 20 μM), KA caused a depolarizing block of ganglion cells. This occurs when a cell is sufficiently depolarized that voltage-dependent sodium channels, once inactivated, remain so and no further action potentials are generated. In extracellular recordings this may be observed as continuous high-frequency firing with a gradual reduction in amplitude until action potentials cease. A depolarizing block of ganglion cells was also caused by 1 mM (or occasionally 500 μM) NMDLA. Therefore, these concentrations, 50 μM-KA or 1 mM-NMDLA, represent an overdose for inner retinal neurones which may cause cell damage.

Quisqualate and AMPA had variable effects on rabbit ganglion cells; 51% (28/55) were excited by QQ, 31% (17/55) were inhibited and 18% (10/55) gave a mixed response. AMPA caused excitation in 73% (8/11) and inhibition in 18% (2/11) of ganglion cells. These compounds were similar in potency to KA for both excitation and inhibition. Dose–response curves plotted for the excitatory effects showed that AMPA was approximately twice as potent as QQ (Fig. 3). The effect of AMPA was very similar to QQ such that the action of QQ, inhibitory or excitatory, always predicted the effect of AMPA. There were no cases where AMPA and QQ had opposite effects on ganglion cells. We could find no obvious correlation between the cell type and effects of QQ or AMPA; at different times excitation and inhibition were observed in 'on' cells and 'off' cells and 'on–off' directionally selective ganglion cells.

We were surprised to find that QQ often caused inhibition, an effect not observed with KA or NMDLA. As a control for variability between different retinae, we purposely applied QQ between pulses of KA and NMDLA which both caused a large increase in firing rate (Fig. 4*A*). In contrast, QQ inhibited the light-evoked response of this cell and reduced the background activity. It is clear that the cell was not rapidly forced into a depolarizing block because there was no sign of excitation and the cell was still firing, albeit at a reduced rate.

Further experiments provide evidence that the inhibitory action of QQ may be polysynaptic, as first suggested by Miller & Coleman (1985). Co-perfusion with the GABA antagonist, picrotoxin, and the glycine antagonist, strychnine, changed the effect of QQ from inhibition to excitation (Fig. 4*B*). This implies that QQ stimulates an inhibitory interneurone which feeds forward to the ganglion cell, masking any direct excitatory influence. In support of this hypothesis, perfusion with QQ elicited action potentials from some ganglion cells when synaptic transmission was blocked with cobalt. This indicates that QQ may have direct excitatory effects on ganglion cells even though previous applications in normal medium caused a reduced firing rate. In addition, the inhibitory effect of QQ on 'off' ganglion cells was converted to excitation in the presence of 2-amino-4-phosphonobutyrate (APB). APB is well known to block 'on' pathways in the retina (Slaughter & Miller, 1981, 1985) but it

also excites many 'off' ganglion cells by disinhibition via glycinergic interneurones (Massey, Redburn & Crawford, 1983; Wassle, Schafer-Trenkler & Voigt, 1986). When this inhibitory input was blocked by APB, QQ caused an increase in ganglion cell firing rate even though control applications in the absence of APB appeared to be inhibitory. Again, this indicates that the inhibitory effects of QQ and AMPA may be polysynaptic in origin.

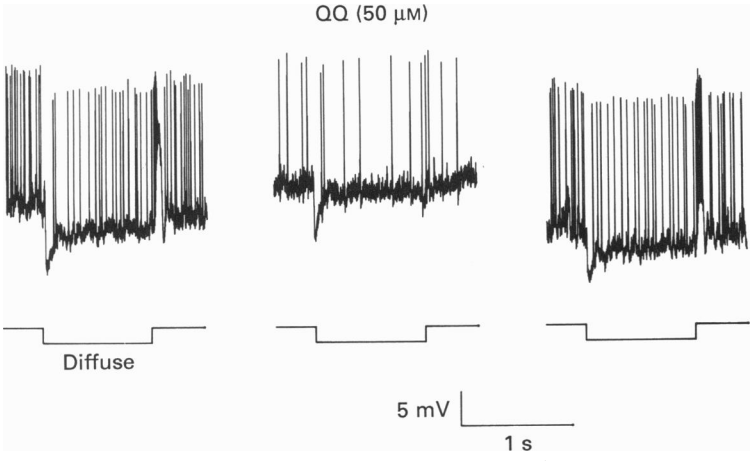

Fig. 5. An intracellular recording from an 'off' ganglion cell; other records from this cell are shown in Figs 1*A* and 11. Note sustained IPSP with fewer action potentials during light stimulus and EPSP with fused spikes at light 'off'. Perfusion with 20 μM-QQ caused a small depolarization, a reduction in firing rate and a reduction in amplitude of the sustained IPSP. These changes are consistent with a large decrease in input resistance, possibly due to the stimulation of an inhibitory interneurone by QQ. The action potentials were attenuated by the recording system. QQ was applied for 45 s; 30 s elapsed between first control and QQ response; 40 s until recovery.

Intracellular recording from ganglion cells also revealed changes that are consistent with a polysynaptic effect for QQ. Figure 5 shows that perfusion with 20 μM-QQ caused a slight depolarization but the cell generated fewer action potentials. The drop in membrane potential was not sufficient to cause a depolarizing block, so the reduction in firing rate probably results from shunting inhibition. In addition, the amplitude of the sustained inhibition caused by light stimulation was reduced. Since this component should increase in amplitude with depolarization (cf. the effect of KA, Fig. 1*A*), this also indicates a large decrease in input resistance, possibly due to stimulation of inhibitory amacrine cells by QQ. In summary, our results from both intracellular and extracellular recording suggest that the variable effects of QQ and AMPA are due to polysynaptic actions.

Antagonists

Recently several glutamate antagonists have been developed which fall roughly into two groups. (i) General antagonists such as cis-2,3-piperidine dicarboxylic acid (PDA) and kynurenic acid (Watkins & Evans, 1981; Perkins & Stone, 1982; Robinson, Anderson & Koerner, 1984). These compounds are effective against multiple glutamate receptors but not very specific within this group. Thus, PDA and kynurenic acid are most potent against NMDA but they also block the action of KA.

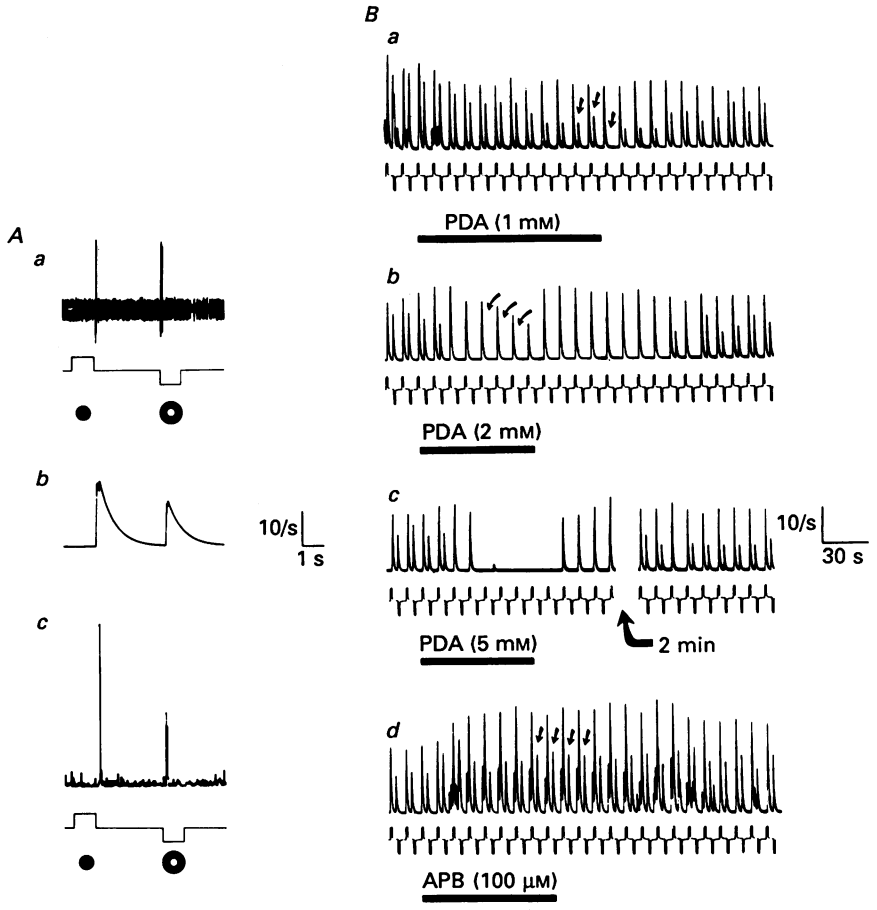

Fig. 6. In this figure, raw data is shown and the cell type is established to the left. Ratemeter records illustrating drug effects are shown on the right. *A a*, an extracellular recording from a brisk transient 'off' cell. This cell gave an 'off' response to a small spot and a surround response to an annulus. A small-amplitude cell with high spontaneous activity was present in the background but the two cells were easily discriminated. *A b*, a ratemeter record of this cell on the same time scale. *A c*, a peristimulus time histogram for this cell; $n = 8$, bin width 20 ms. *B a*, a ratemeter record showing the response to perfusion with 1 mM-PDA. Spontaneous activity was abolished and the surround response to annular stimulation was preferentially reduced (arrows). *B b*, 2 mM-PDA selectively blocked the surround responses (arrows). *B c*, 5 mM-PDA blocked all light-evoked activity. The centre response recovered first followed by the surround response after a delay of 2 min. *B d*, the response of the same cell to 100 μM-APB. APB potentiated the light responses of this cell and did not block the surround response (arrows). Calibration bars in this figure and in Figs 7–10 indicate the cells' firing rate in action potentials per second and the time scale.

(ii) NMDA antagonists such as 2-amino-7-phosphonoheptanoate (AP-7) (Davies, Francis, Jones & Watkins, 1981). These compounds are both potent and specific and may be used to differentiate between KA and NMDA receptors. In the rabbit retina, NMDA antagonists have only minor effects on the light-driven activity of amacrine and ganglion cells (Massey & Miller, 1985*b*).

We have repeatedly observed that general glutamate antagonists such as PDA and kynurenic acid block the light-evoked input to all types of ganglion cells in the rabbit retina. The potency of these compounds is notoriously low, so initially we compared several concentrations to find an effective dose. Figure 6 illustrates the ratemeter records from an extracellular recording from a transient 'off' ganglion cell. Centre

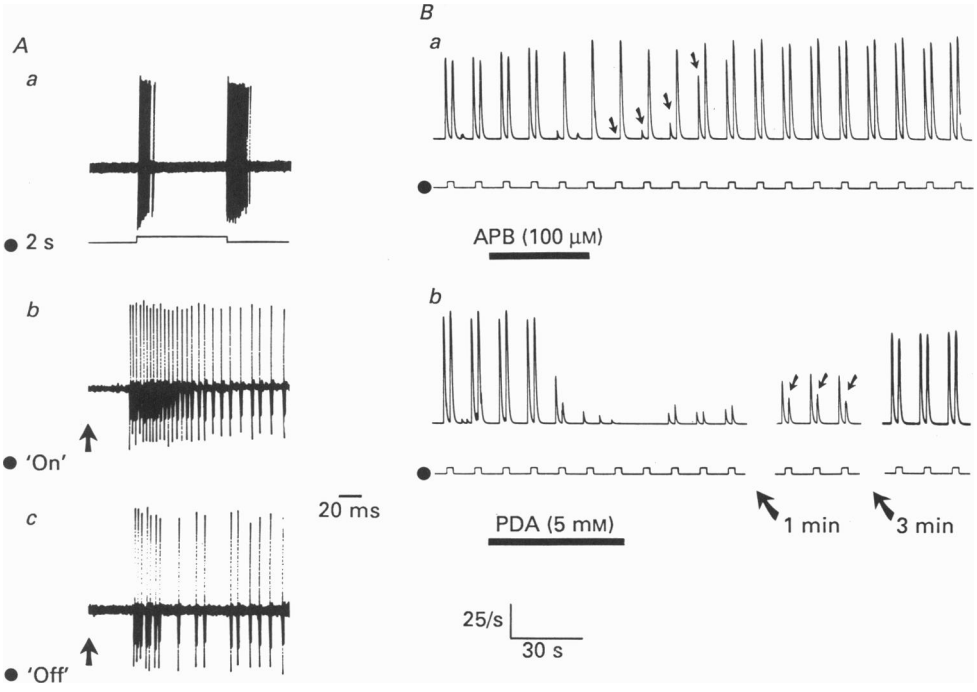

Fig. 7. *A a*, extracellular data show this cell gave an 'on–off' response to a small spot. The time scale is shown by the length of the stimulus, 2 s. The two lower traces (*A b* and *c*) were taken at an expanded time scale (calibration bar, 20 ms), triggered at light 'on' or light 'off', to show that both the 'on' and 'off' responses are short latency (50 ms) as opposed to the long latency (100 ms) responses generated by antagonistic surrounds. Although directional selectivity was not demonstrated, this cell, recorded in the periphery, was very probably an 'on–off' directionally selective ganglion cell. *B a*, a ratemeter record showing that perfusion with APB selectively blocked the 'on' response of this cell. On wash-out the 'on' component gradually regained its former amplitude (arrows). The light stimulus was a small spot. *B b*, PDA reversibly blocked both 'on' and 'off' responses to light stimulation. The 'off' responses were slower to recover (arrows).

and surround responses to a spot and annulus were established and then increasing doses of PDA added to the perfusate. At 1 mM, PDA blocked the spontaneous activity and preferentially reduced the surround responses elicited by annular stimulation. Surround responses were reversibly abolished by 2 mM-PDA and the centre responses to a small spot were reduced. When the dose of PDA was raised to 5 mM, all light-driven responses were blocked until the drug was washed out and control responses were obtained once more. A similar gradation of effects was obtained with kynurenic acid, but it was slightly more potent. In contrast, APB which selectively blocks 'on' pathways in the vertebrate retina, did not block the

light-evoked responses of this cell. In fact, both centre and surround responses were potentiated by APB. The results of Fig. 6 indicate that (i) PDA can block the light input to 'off' ganglion cells; (ii) an effective but reversible dose for PDA is 5 mM; and (iii) APB and PDA act at different sites in the retina.

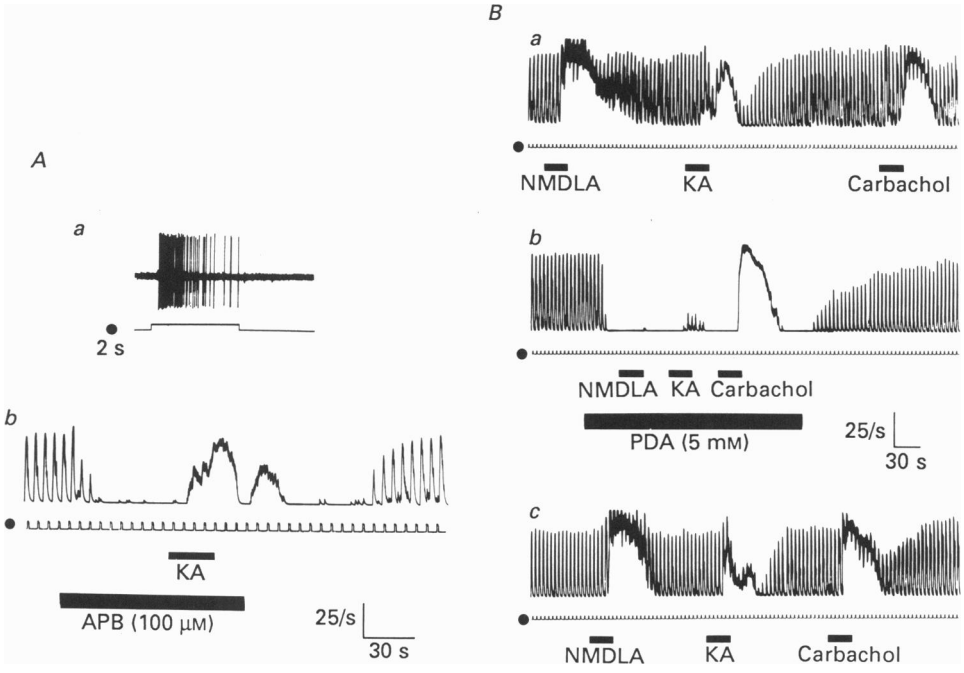

Fig. 8. *A a*, an extracellular recording which shows an 'on' response to a small spot. The time scale is indicated by the length of the stimulus, 2 s. *A b*, a ratemeter record which shows that the 'on' response to a small spot is blocked by APB. However, APB does not block the action of KA. This is because KA has a direct excitatory action on ganglion cells whereas APB blocks 'on' channels by an action at a different site in the outer retina. *B*, a continuous ratemeter record showing the pharmacological selectivity of PDA. *B a*, control responses to NMDLA (400 μM), KA (200 μM) and carbachol (40 μM), all of which caused a large increase in firing rate. *B b*, perfusion with PDA eliminated the light-evoked response of this cell and blocked the action of NMDLA and KA. The excitation due to carbachol was not affected. *B c*, recovery; control responses were obtained after the wash-out of PDA. The calibration bar applies to all ratemeter records.

A further comparison between PDA and APB was made for the 'on–off' cell illustrated in Fig. 7. As previously reported APB selectively blocked the 'on' response of this cell but the 'off' responses remained. In contrast, PDA blocked both 'on' and 'off' responses from this cell. There is some indication that 'off' responses may be more sensitive to PDA because they were abolished first and subsequently they were slow to recover.

The interpretation of results obtained using PDA depends critically on its pharmacological specificity. Therefore, we compared the effect of PDA against glutamate analogues and a cholinergic agonist, carbachol. In control responses NMDLA, KA and carbachol all caused a large increase in the firing rate of the 'on' ganglion cell shown in Fig. 8. Perfusion with 5 mM-PDA blocked the light responses

of this cell, abolished the effect of NMDLA and greatly reduced the effect of KA. However, the excitation due to carbachol was not affected by PDA. Since KA and NMDLA can both excite ganglion cells directly, this suggests that PDA can block ganglion cells by a direct antagonist action. Recovery of the light activity and control responses to KA, NMDLA and carbachol were obtained after wash-out. In

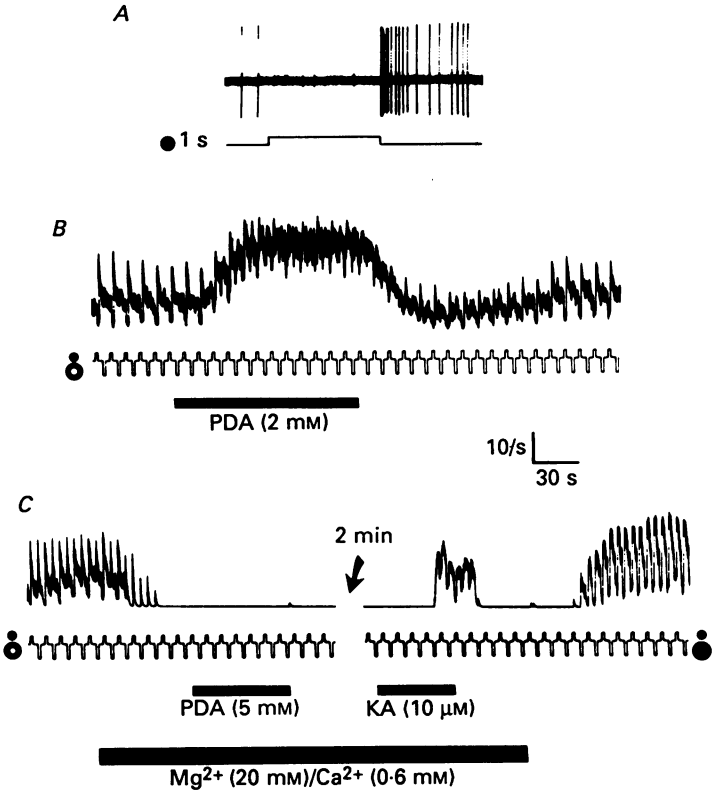

Fig. 9. The excitatory action of PDA on 'off' ganglion cells is indirect. *A*, an extracellular recording showing an 'off' response to a small spot. The time scale is indicated by the length of the stimulus trace. *B*, a ratemeter record from the same cell showing alternate responses to a spot and annulus. PDA increases the background firing rate but the light-evoked responses are almost abolished. *C*, during perfusion with high Mg^{2+}/low Ca^{2+} to block synaptic transmission, a high dose of PDA had no effect. As a control KA caused a strong excitation when applied during the synaptic block. This indicates that PDA does not excite ganglion cells directly.

addition, we compared the effect of APB on the activity of this cell evoked by light and KA. As expected APB reversibly blocked the light input to this 'on' ganglion cell but it did not antagonize the effect of KA. This is because APB blocks 'on' channel activity by interaction with the photoreceptor–'on' bipolar cell synapse in the outer retina whereas KA excites ganglion cells directly. Thus, although APB and PDA can both block 'on' ganglion cells, they do so by action at different sites; APB in the outer retina and PDA in the inner retina. In summary, the results in Fig. 8 indicate that (i) PDA blocks the light input to 'on' ganglion cells, (ii) PDA is a

selective antagonist against certain glutamate analogues, and (iii) PDA acts at a different site than APB and has a direct antagonist action on ganglion cells.

We also observed that PDA often excited 'off' ganglion cells as shown in Fig. 9. Although the light-driven input to this cell was almost abolished by 2 mM-PDA, the background firing rate increased dramatically. However, when synaptic transmission

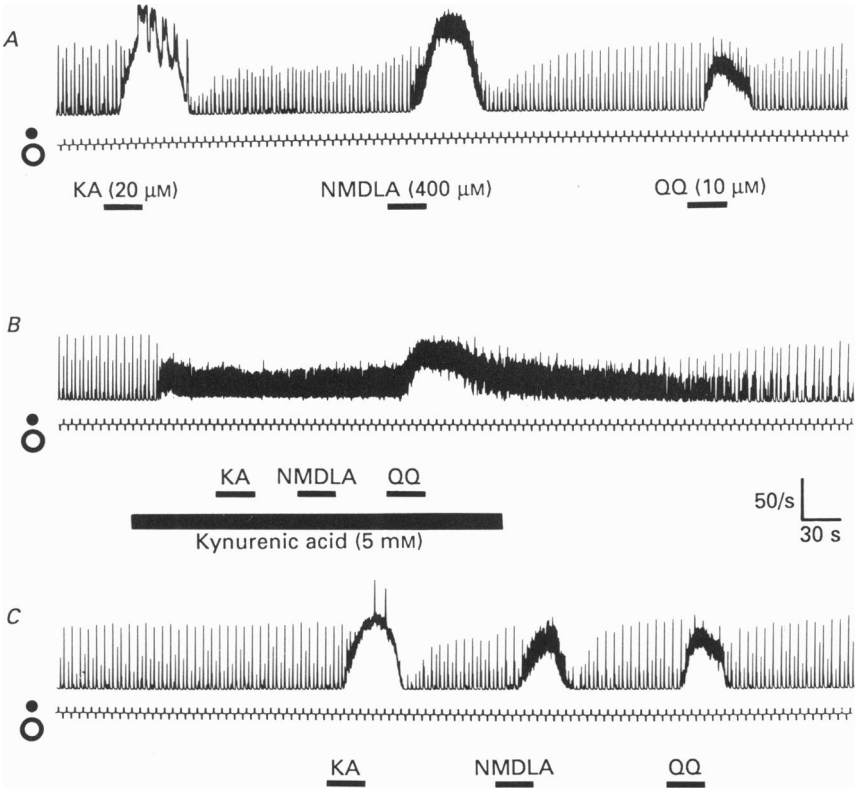

Fig. 10. A continuous record from a large field unit. This cell was identified by a strong dimming response, preference for rapidly moving stimuli, and strong centre–surround response to a diffuse light. A small spot which gave an 'off' response and an annulus which gave a surround response at light on were presented in succession. *A*, control response to KA, NMDLA and QQ, all of which caused a large increase in firing rate. *B*, perfusion with kynurenic acid caused an increase in the background firing rate, probably due to disinhibition, and blocked the light-evoked response. The effects of KA and NMDLA were abolished but the excitatory action of QQ persisted. Thus it is unlikely that QQ receptors mediate the light-evoked input to ganglion cells. *C*, recovery, light responses and control responses to KA, NMDLA and QQ were obtained after the wash-out of kynurenic acid.

was blocked with high magnesium–low calcium, a higher dose of PDA had no effect. The cell was still capable of firing under these conditions since KA, which excites ganglion cells directly, elicited a strong burst of action potentials. This suggests that the excitatory action of PDA on 'off' ganglion cells is indirect, perhaps due to disinhibition from an intermediary cell. APB, which blocks 'on' pathways in the outer retina, also excites 'off' ganglion cells, probably due to the block of an intermediary glycinergic amacrine cell which receives input from an 'on' bipolar cell

(Wassle *et al.* 1986). Preliminary experiments show that the excitatory effects of strychnine and kynurenic acid on 'off' ganglion cells are *not* additive. This supports the idea that glutamate antagonists excite 'off' ganglion cells due to the block of a glycinergic interneurone.

It has previously been reported that kynurenic acid blocks the action of KA but

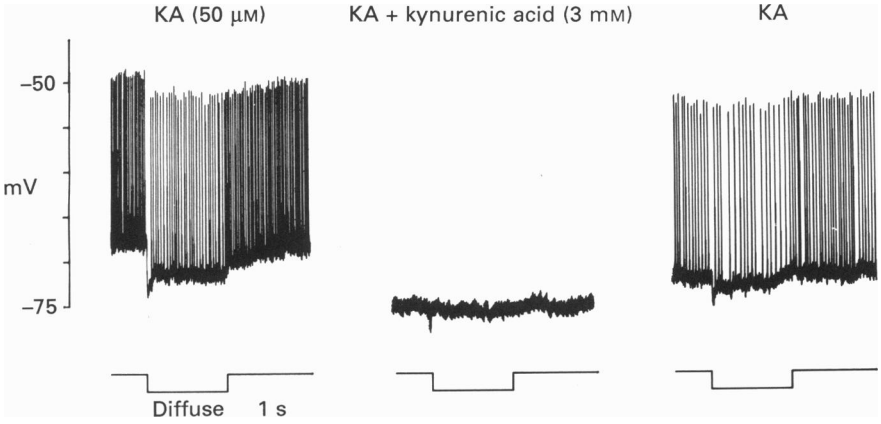

Fig. 11. Sections from a continuous intracellular recording from an 'off' ganglion cell. The time scale is indicated by the length of the stimulus. The same cell is shown in Figs 1*A* and 5. Perfusion with kynurenic acid hyperpolarized this cell by approximately 5 mV and the action potentials stopped (not shown). The cell remained quiescent after kynurenic acid was washed out. Subsequent perfusion with KA depolarized the cell and elicited a chain of action potentials (left). As KA washed out, the cell hyperpolarized once more and the cell did not fire. Centre, perfusion with kynurenic acid caused no change in membrane potential but completely blocked the depolarization and action potentials elicited by KA. Right, recovery, 2 min after kynurenic acid, KA caused a small depolarization but did not pass the spike threshold (not shown). After 5 min, KA caused a larger depolarization and generated a train of action potentials once more.

not QQ in the mudpuppy retina (Coleman *et al.* 1986). Since this represents a way to differentiate between these receptor subtypes, we tested kynurenic acid in the rabbit retina (Fig. 10). The doses of KA, NMDLA and QQ were adjusted so that each agonist caused a comparable increase in firing rate. On perfusion with kynurenic acid, the spontaneous activity increased but the light input was blocked. This is very similar to the indirect excitation seen with PDA and probably results from disinhibition. Kynurenic acid abolished the effect of NMDLA and KA, but QQ still caused a large increase in firing rate. Recovery of the light responses and control responses to KA, NMDLA and QQ were obtained on wash-out. The data in Fig. 10 indicate that although kynurenic acid blocked the light-driven activity of this cell, responses to QQ persisted. Thus, it is unlikely that the light-driven responses of ganglion cells are mediated by QQ receptors.

Intracellular recording indicates that kynurenic acid blocks the depolarization induced by KA as well as the increased firing rate. The records in Fig. 11 are representative sections taken from a much longer trace recorded from an 'off' ganglion cell. At first the cell fired normally and was light driven, but perfusion with kynurenic acid caused a small hyperpolarization and no further action potentials were generated. This is not shown because recovery was not obtained on wash-out.

However, perfusion of this cell with 20 μM-KA caused a 7 mV depolarization and elicited a prolonged burst of action potentials. Once the cell passed the spike threshold it can be clearly seen that it was light driven again. This effect was reversible, so when the KA washed out, the cell hyperpolarized and stopped firing. Perfusion with kynurenic acid (3 mM) caused no visible change in the resting

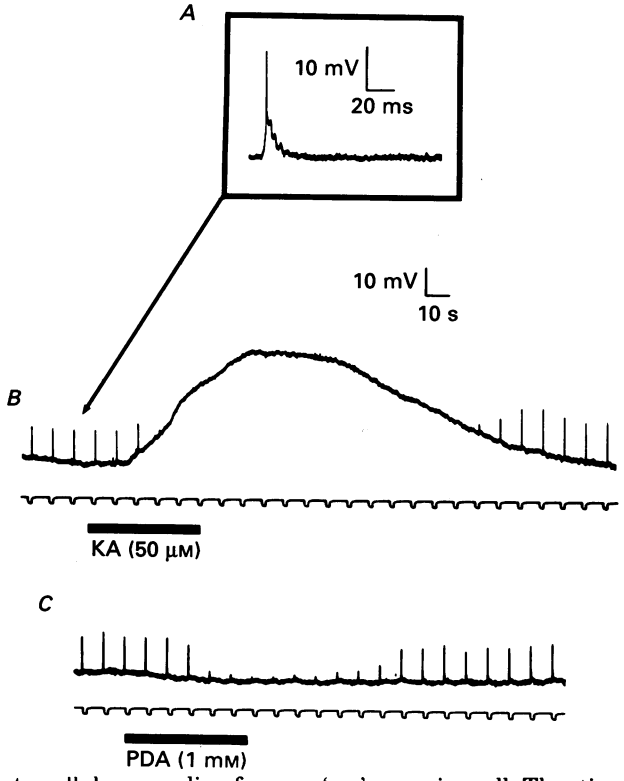

Fig. 12. An intracellular recording from an 'on' amacrine cell. The stimulus was a diffuse light. *A*, an oscilloscope trace showing the cell's response on an expanded time scale. Light onset produced one or two small action potentials riding on an EPSP. This is typical of an amacrine cell response. *B*, a pen recorder trace for the same cell; the light-evoked responses are attenuated by approximately 50%. The resting membrane potential was −75 mV. Perfusion with a large dose of KA (50 μM) caused a reversible depolarization of 40 mV. *C*, perfusion with a low dose of PDA (1 mM) slightly hyperpolarized the cell and almost abolished the light-evoked response. Control responses were obtained on wash-out.

membrane potential but completely blocked the effect of KA. After kynurenic acid was washed out, a control response to KA was obtained. This indicates that kynurenic acid had no direct effect on ganglion cells, but reversibly blocked the depolarization and increased firing rate associated with KA.

Amacrine cells

Amacrine cells are also third-order neurones which are postsynaptic to bipolar cells at ribbon synapses in the inner plexiform layer. Intracellular recordings from amacrine cells were identified by their physiological responses: typically, they have

prominent EPSPs at light 'on' or 'off' with one or two small action potentials superimposed. Amacrine cells are also extremely sensitive to glutamate analogues. An example from an 'on' amacine cell is shown in Fig. 12. Brief perfusion with a high dose of KA (50 μM) caused a massive depolarization of 40 mV. At this concentration, which we consider to be an overdose for the inner retina, KA invariably caused continuous firing in ganglion cells followed by a depolarizing block. It is not surprising that such large changes in membrane potential with associated ionic movements are toxic to retinal neurones. Results from other amacrine cells, 'on' and 'off', indicate that the action of KA on amacrine cells persists when light responses are blocked by high magnesium. Therefore, the effect of KA on amacrine cells should be considered direct. Similar excitatory effects on amacrine cells were seen with QQ and NMDLA, but as with ganglion cells, NMDLA was much less potent. Inhibitory effects of QQ on amacrine cells were not observed. Finally, perfusion with PDA caused a small hyperpolarization and reversibly inhibited the light-evoked responses of the 'on' amacrine cell in Fig. 12. Amacrine cell responses were not affected by NMDA antagonists such as AP-7, so we conclude that the glutamate receptors of amacrine cells are probably KA (or QQ) receptors similar to those of ganglion cells.

DISCUSSION

Using a combination of extracellular and intracellular recording, we have analysed the effects of various glutamate analogues and antagonists on amacrine and ganglion cells of the perfused rabbit retina. Our results lead to three major conclusions: (i) all ganglion cells are directly excited by glutamate analogues, especially KA and NMDLA; (ii) the light-driven activity of all ganglion cells is blocked by general glutamate antagonists such as PDA and kynurenic acid; (iii) the light-driven input to ganglion cells is mediated predominantly by KA receptors; this is consistent with the idea that 'on' and 'off' bipolar cells use glutamate as a neurotransmitter.

Agonists

KA had a direct excitatory action on third-order neurones and produced a regular train of action potentials from ganglion cells. In contrast, NMDLA elicited burst firing from ganglion cells not typical of light-driven activity. The variable effect of QQ, sometimes excitatory, but often inhibitory, was surprising because glutamate analogues almost invariably excite neurones in the CNS by opening a non-selective cation channel (Mayer & Westbrook, 1987). Our initial doubts about this result were partly alleviated when we found identical results with AMPA, an ibotenic acid derivative which appears to recognize the QQ site in binding studies (Foster & Fagg, 1984; Olsen, Szamraj & Houser, 1987). Since the inhibitory effects of QQ and AMPA are reversed by treatments which block the inhibitory input to ganglion cells, it may be that QQ and AMPA have polysynaptic actions, the sum of which results in net inhibition. However, the block of inhibitory input may be expected to raise the input resistance of ganglion cells and this factor alone may potentiate the excitatory effects of QQ and AMPA. Still, it is puzzling that KA, which clearly depolarizes amacrine cells, never inhibited ganglion cells. One explanation could be that some amacrine cells respond preferentially to QQ although neither we nor Bloomfield & Dowling

(1985) observed a marked difference between QQ and KA on amacrine cell responses.

Alternatively, QQ may be less effective on some ganglion cells due to desensitization. This is a progressive reduction in response which occurs in the presence of agonists, while the receptor is still occupied. Desensitizing responses to QQ have been reported for retinal horizontal cells (Ishida & Neyton, 1985) and ganglion cells (Aizenman, Frosch & Lipton, 1988) but KA causes a prolonged non-desensitizing response. The methods used in this study were not designed to investigate these questions, but more detailed information may be expected from patch clamp techniques.

In summary, our agonist studies show that NMDA receptors are not likely to mediate the light-evoked input to ganglion cells. The effects of KA and QQ were often different. However, this unspecified difference does not allow us to rule out QQ receptors, since clearly the bipolar cell transmitter can be expected to stimulate amacrine cells and generate polysynaptic effects. All these receptor types may be activated by glutamate, so their presence on ganglion cells is consistent with the release of glutamate from 'on' and 'off' bipolar cells.

Antagonists

The ability of PDA and kynurenic acid to block responses to KA and NMDLA, but not carbachol, indicates that they are pharmacologically selective glutamate antagonists in the rabbit retina. However, their effects are widespread throughout the retina and interpretation of our results depends critically on their site of action. 'On' bipolar cells, which are light driven via the APB receptor, are not affected by PDA in the mudpuppy retina (Slaughter & Miller, 1983*a*). This is presumed to be the case in the rabbit retina since PDA reduced, but did not block, the b-wave of the ERG, a second-order response reflecting 'on' bipolar cell activity (Cunningham & Neal, 1985). Therefore, since PDA blocks 'on' responses of third-order neurones, which receive direct input from 'on' bipolar cells, we deduce that PDA blocks the effect of the 'on' bipolar cell transmitter. This implies that 'on' bipolar cells release an excitatory transmitter such as glutamate. The analysis is more difficult for 'off' pathways since PDA and kynurenic acid block 'off' bipolar cells in the outer retina (Slaughter & Miller, 1983*a*; Coleman *et al.* 1986). However, we note that 'off' responses of 'on–off' ganglion cells appear to be more sensitive to PDA. Furthermore, since PDA and kynurenic acid block exogenous NMDLA or KA they must have antagonist activity directly on 'off' ganglion cells. Thus, it is likely that glutamate antagonists do block the effect of the 'off' bipolar cell transmitter. A similar analysis using PDA was first reported for the mudpuppy retina (Slaughter & Miller, 1983*c*).

PDA and kynurenic acid are general glutamate antagonists which block the effects of both KA and NMDLA in the rabbit retina. In contrast NMDA antagonists, such as AP-7, are extremely specific and may be used to differentiate between KA and NMDA receptors (Davies *et al.* 1981). However, in the rabbit retina, AP-7 had little effect on ganglion cell responses (Massey & Miller, 1985*b*). Therefore, we deduce that the light input to ganglion cells is not mediated by NMDA receptors. KA and QQ receptors have been more difficult to separate, but excitation due to QQ persisted

when the effects of KA and light input were blocked by kynurenic acid. This has also been reported for the mudpuppy retina and similar activity was found with PDA (Coleman *et al.* 1986). Therefore, the major physiological input from bipolar cells to ganglion cells appears to be mediated by KA receptors.

Further support for the role of glutamate as a bipolar cell transmitter comes from studies on ACh release from the rabbit retina. Cholinergic amacrine cells are known to receive direct input from cone bipolar cells (Famiglietti, 1983) and glutamate or KA causes massive ACh release, up to 50 times the basal rate. Furthermore, the light-evoked release of ACh and the effect of KA were blocked by PDA (Cunningham & Neal, 1985; S. C. Massey, unpublished observations). If PDA does not block the 'on' bipolar cell (Slaughter & Miller, 1983*a*, *c*), which has direct input to 'on' cholinergic amacrine cells, then it must act by antagonizing the bipolar cell transmitter. This indicates that 'on' bipolar cells probably release glutamate. The effect of KA on cells of the inner retina is widespread but not universal: KA does not stimulate dopamine release from carp retina (O'Connor, Dorison, Watling & Dowling, 1986). Interestingly, dopaminergic interplexiform cells in the carp retina receive input primarily from amacrine, but not bipolar, cells (Dowling & Ehinger, 1978).

PDA and kynurenic acid increased the spontaneous activity of some 'off' ganglion cells even though their light-driven activity was abolished. In the presence of magnesium to block synaptic activity, PDA and kynurenic acid had no effect (Fig. 9), so their excitatory action was indirect. This is very similar to the APB-induced excitation of 'off' ganglion cells except, of course, APB did not block the light-evoked activity. The APB effect is due to 'on' disinhibition via a glycinergic interneurone (Wassle *et al.* 1986). Since the excitatory effects of kynurenic acid and strychnine were not additive, the same mechanism may explain the effects of PDA and kynurenic acid. Likely candidates for 'on' to 'off' inhibition are the bistratified amacrine cells A7 and A8 which are known to be glycinergic in the cat retina (Pourcho & Goebel, 1985). APB causes 'on' disinhibition by blocking the 'on' bipolar cell in the outer retina. The analysis presented in this paper suggests that PDA and kynurenic acid may mimic this effect by blocking 'on' bipolar cell output at KA receptors in the inner retina.

Other transmitter candidates for bipolar cells

Acetylcholine also excites many ganglion cells by a direct action (Masland & Ames, 1976), but in the rabbit retina it is restricted to a subpopulation of amacrine cells (Masland & Tauchi, 1986). Some cholinergic cells in the chick retina, first thought to be bipolar cells (Baughman & Bader, 1977), were subsequently identified as amacrine cells (Morgan, Ishimoto & Miller, 1985). Certain neuropeptides may also be excitatory, but again they have only been found in amacrine cells (Brecha, 1983).

Glycine, which is generally regarded as an inhibitory transmitter, has been localized to 10–15% of bipolar cells in the cat retina (McGuire, Stevens & Sterling, 1984; Cohen & Sterling, 1986; Wassle *et al.* 1986; Pourcho & Goebel, 1987) and Sterling (1983) has suggested that some bipolar cells may be inhibitory. However, glycine is found in at least one cone bipolar cell type, CBb_1 or cb5, which is known to be excitatory (Nelson & Kolb, 1983). It is possible that glycine may enter some

bipolar cells via well-described gap junctions with glycinergic amacrine cells (Cohen & Sterling, 1986). However, this has not yet been demonstrated and thus the role of glycine in bipolar cells remains unresolved.

In summary, neither ACh, glycine or peptides are good candidates for the bipolar cell transmitter. In this paper, we have presented evidence that several glutamate receptors are present on ganglion cells and that the physiological responses of third-order neurones are blocked by glutamate antagonists. These data suggest that an excitatory amino acid such as glutamate may be the transmitter for 'on' and 'off' bipolar cells.

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